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Supplemental information

Synonymous codon usage regulates translation initiation

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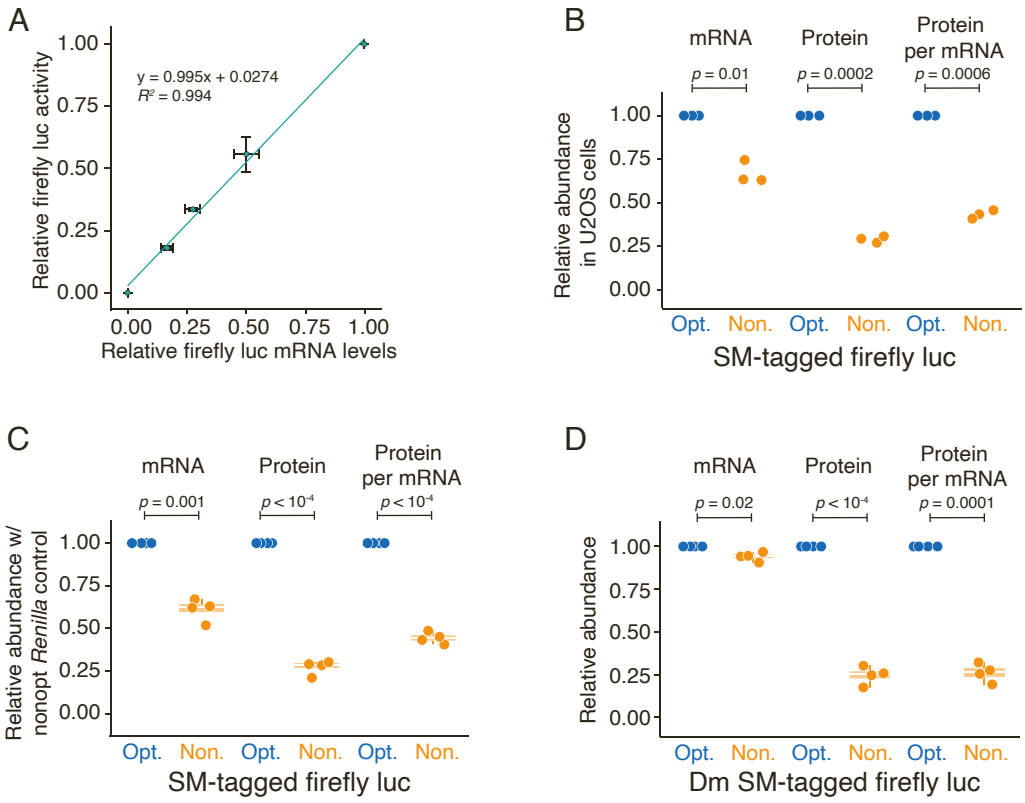


Figure S1. Additional protein effect does not depend on the amount of transfected plasmid, cell line, nor transfection control; and may be conserved in Drosophila cells [Related to Figure 1].

(A) Protein activity from optimal flag-tagged spaghetti monster firefly luciferase reporter change linearly with mRNA levels. HEK293T cells were transfected with various concentrations (0, 0.125, 0.25, 0.5, and 1 ug) of the plasmid expressing the firefly construct and an optimized *Renilla* luciferase. mRNA levels were quantified by RT-qPCR, and protein activity was quantified by dual luciferase assay. A scatterplot shows the relationship between relative mRNA and protein activity. Mean values ± SD are shown; *n*=3. **(B)** In U2OS cells, firefly luciferase optimality reporters show differences in protein output, even when normalized to transcript abundance. U2OS cells were transfected with either Flag-tag spaghetti-monster optimal ("Opt.") or nonoptimal (Non.") firefly luciferase reporters and *Renilla* luciferase, as a transfection control. mRNA levels were quantified by RT-qPCR, and protein activity was quantified by dual luciferase assay. Shown are scatter plots for the fold-change of the nonoptimal reporter relative to the optimal one for mRNA abundance, protein abundance, and normalized protein-per-mRNA abundance. *P*-values were determined using paired Student's t-tests; *n*=3. **(C)** Additional protein level effect does not depend on co-transfected optimized *Renilla* luciferase. HEK293T cells were transfected with either Flag-tag spaghetti-monster optimal ("Opt.") or nonoptimal (Non.") firefly luciferase reporters and nonoptimal, wildtype *Renilla* luciferase, as a transfection control. Relative mRNA and protein activity were determined, and plotted, as in S1B. *P*-values were determined using paired Student's t-tests; *n*=4. **(D)** Nonoptimal codons repress protein output in *Drosophila*. S2 cells were transfected with plasmids expressing either λHA-tagged optimal "Opt." or nonoptimal "Non." firefly luciferase, with *Renilla* luciferase as a co-transfection control. Relative mRNA and protein activity were calculated and plotted, as in S1B; *n*=4.

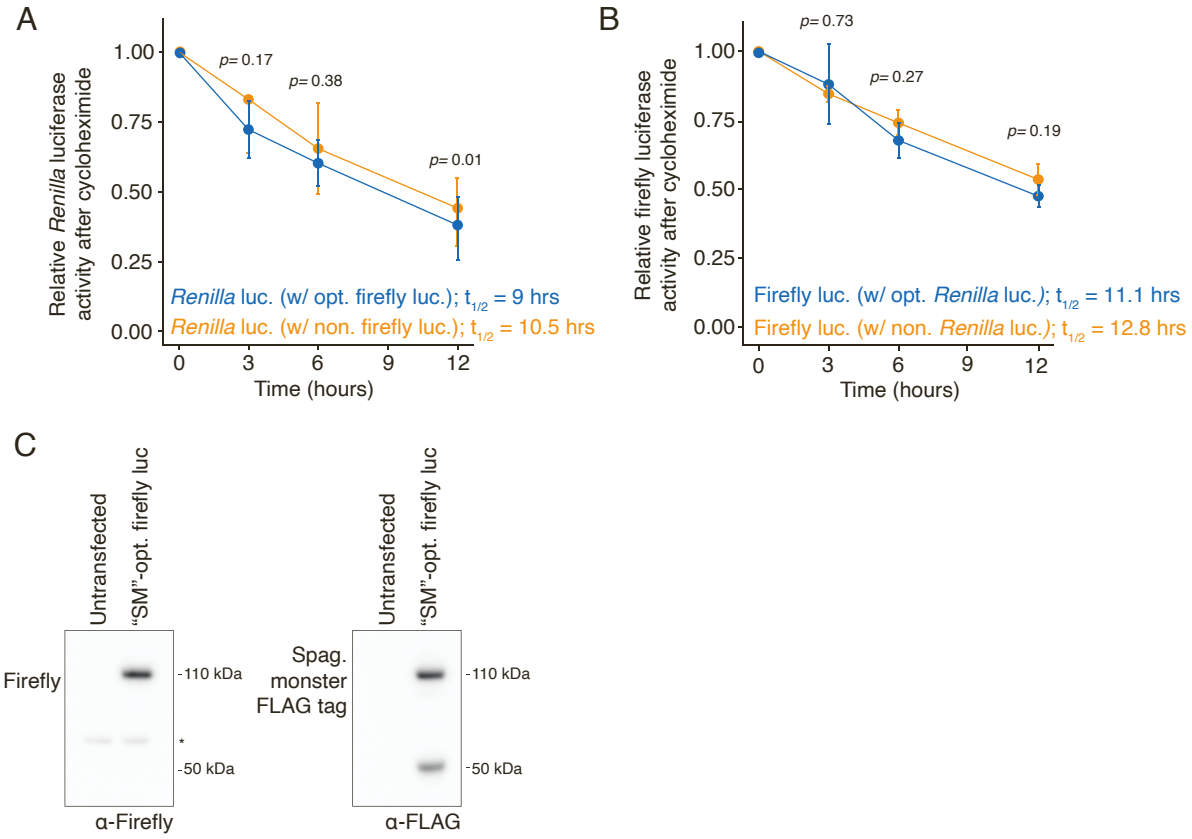


Figure S2. The cycloheximide time course shows no difference in the half-lives of either co-transfection control, and the flag-tagged spaghetti monster peptide is cleaved [Related to Figure 2].

(A) Optimized *Renilla* luciferase co-transfection controls from Figure 2B have no significant difference in functional half-life. Cells were co-transfected with the spaghetti-monster tagged firefly luciferase reporters and optimized *Renilla* luciferase, and then harvested at various time points following cycloheximide treatment. The amount of *Renilla* luciferase control was determined by luciferase assay and normalized to the initial (0 hr) time point. *P*-values were determined using paired Student's t-tests. Mean values ± SD are shown; *n*=3. **(B)** Optimized firefly luciferase co-transfection controls from Figure 2C have no significant difference in functional half-life. Cells were co-transfected with the spaghetti-monster *Renilla* luciferase reporters and optimized firefly luciferase, and then harvested at various time points following cycloheximide treatment. The amount of functional firefly luciferase control was determined by luciferase assay and normalized to the initial (0 hr) time point. *P*-values were determined using paired Student's t-tests. Mean values ± SD are shown; *n*=3. **(C)** The lower bands marked by an asterisk (*) in Figures 2D and 2E refer to the cleaved flag-tagged spaghetti monster. Lysates expressing the optimized flag-tagged spaghetti-monster firefly luciferase gene were probed with both α-firefly and α-Flag. The lower band (~50 kDa) is only detectable with the α-Flag antibody. The band marked with an asterisk (*) in this α-firefly blot is a nonspecific band at ~60 kDa.

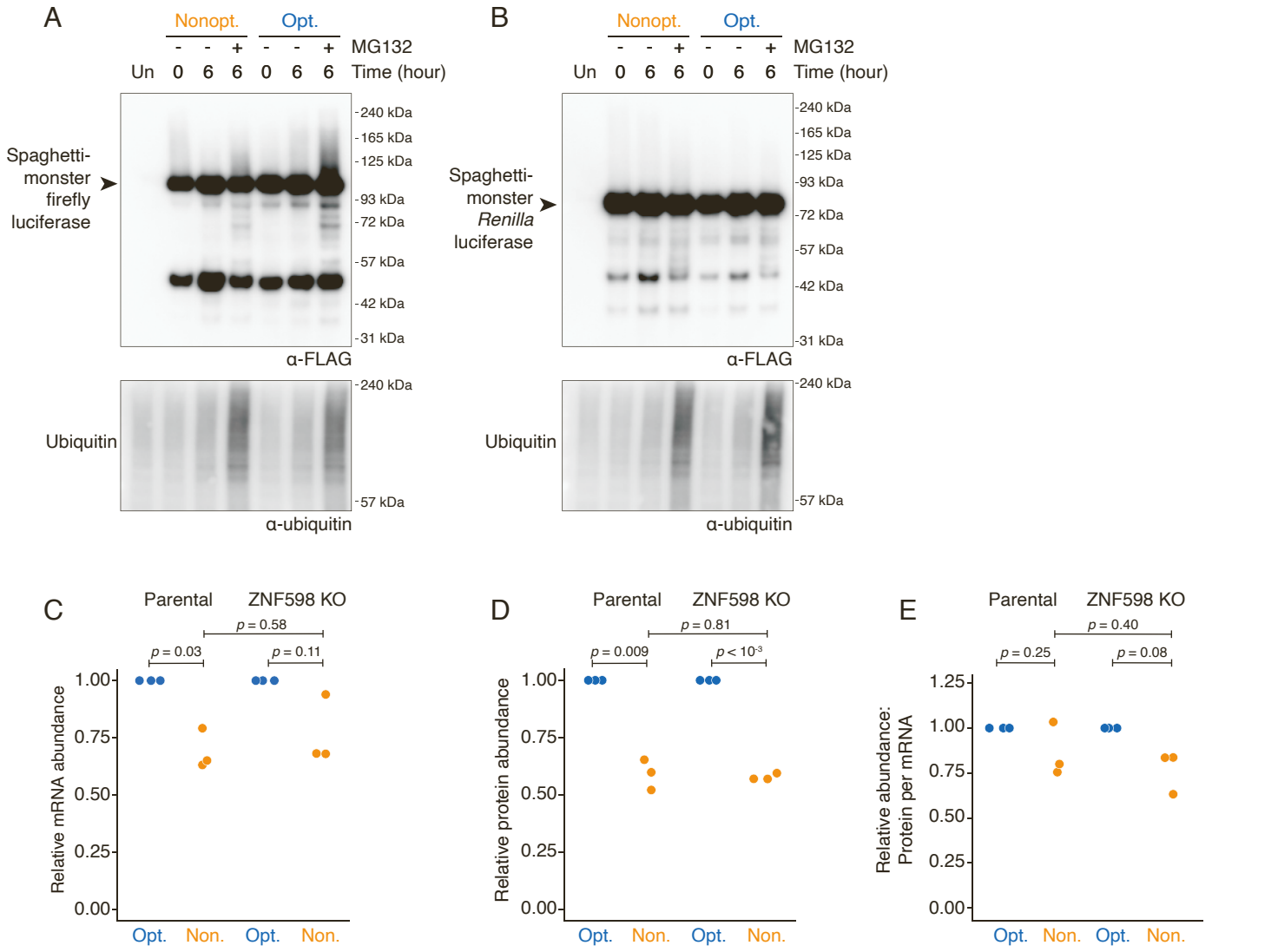


Figure S3. Nonoptimal synonymous codon usage of luciferase reporters do not uniquely accumulate truncated protein products, and ZNF598 plays no role in modulating mRNA or protein levels of reporters [Related to Figures 3 & 4].

(A) Nonoptimal codons do not lead to truncated protein products. HEK239T cells were transfected with spaghetti-monster firefly luciferase reporters and treated with MG132 (Millipore) or DMSO for six hours. Western blotting was performed, probing for the firefly luciferase reporter (α-Flag) and for ubiquitin. Twice the volume of lysate was loaded for samples with nonoptimal firefly luciferase. Shown is an overexposed blot to detect the accumulation of truncated protein products. **(B)** As in A, except for the spaghetti-monster *Renilla* luciferase reporters. Twice the volume of lysate was loaded for samples with nonoptimal *Renilla* luciferase. **(C)** ZNF598 does not play in role in modulating nonoptimal mRNA levels. Data are from the same experiment as Figure 4F. Shown are dot plots for the fold change of normalized mRNA abundance of the nonoptimal reporter, with optimal mRNA levels normalized to one. The *p*-value was determined using a paired Student's t-test; *n*=3. **(D)** ZNF598 does not play a role in modulating nonoptimal protein levels. Data are from the same experiment as Figure 4F and S3C. Shown are dot plots for the fold change of normalized protein activity, as determined by dual luciferase activity, with optimal protein levels normalized to one. The *p*-value was determined using a paired Student's t-test; *n*=3. **(E)** ZNF598 does not play a role in repressing nonoptimal protein expression. Parental Flp-In HEK293T cells, with or without ZNF598 (Juszkiewicz and Hegde, 2017) [1], were maintained in tetracycline-free media to keep the previously integrated stall reporter from being expressed. Cells were transfected with plasmids expressing flag-tag spaghetti-monster firefly luciferase optimality reporters. Shown are dot plots for the fold change of normalized functional protein-per-mRNA abundance of the nonoptimal reporter relative to the optimal one. The *p*-values were determined using a paired Student's t-test; *n*=3.

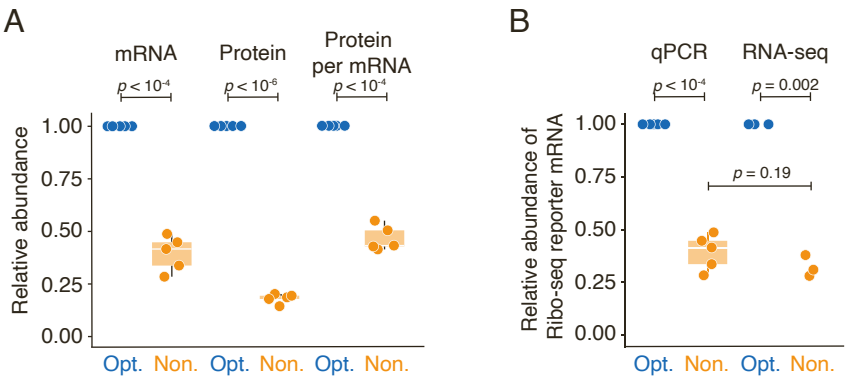


Figure S4. Ribo-seq reporters show additional protein-level effect, for which frameshifting is not responsible [Related to Figure 4].

(A) Dual-tagged Ribo-seq optimality reporters show differences in firefly luciferase activity, even after controlling for transcript levels. HEK293T cells were transfected with either nonoptimal (Non.) or optimal (Opt.) firefly luciferase, tagged at the N-terminus with the spaghetti-monster tag, and tagged at the C-terminus with optimized GFP. Transcript abundance was quantified by RT-qPCR, and protein activity was quantified by dual luciferase assay. Shown are scatter plots for the fold-change of the nonoptimal reporter relative to the optimal one for mRNA abundance, protein abundance, and normalized protein-per-mRNA abundance. *P*-values were determined using paired Student's t-tests; *n*=5. **(B)** Nonoptimal codons reduce the mRNA abundance of the dual-tagged reporters. mRNA abundance for each reporter was determined by RT-qPCR or RNA-seq (from the same samples used for the Ribo-seq libraries) and normalized to the *Renilla* luciferase co-transfection control. RNA-seq reads spanning across the N-terminal spaghetti monster tag were used. *P*-value was determined by unpaired Student's t-test. qPCR: *n*=5; RNA-seq: *n*=3.

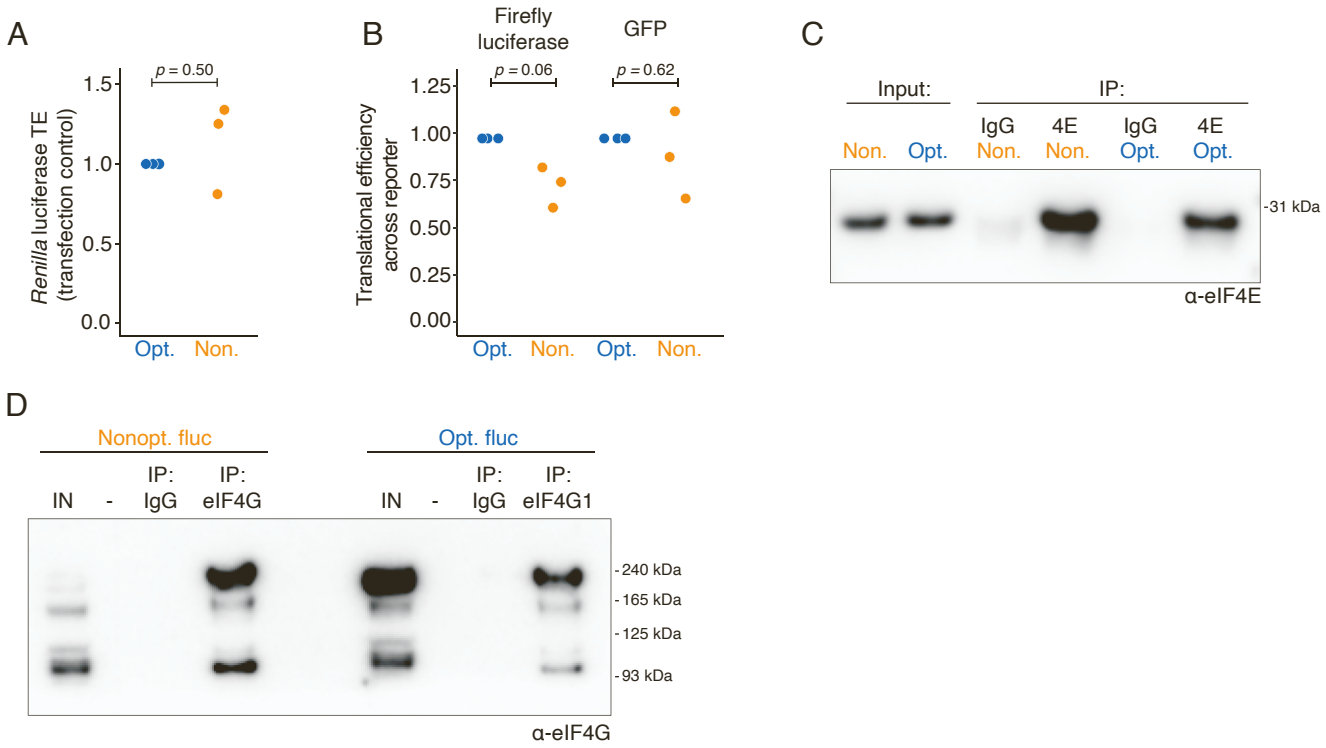
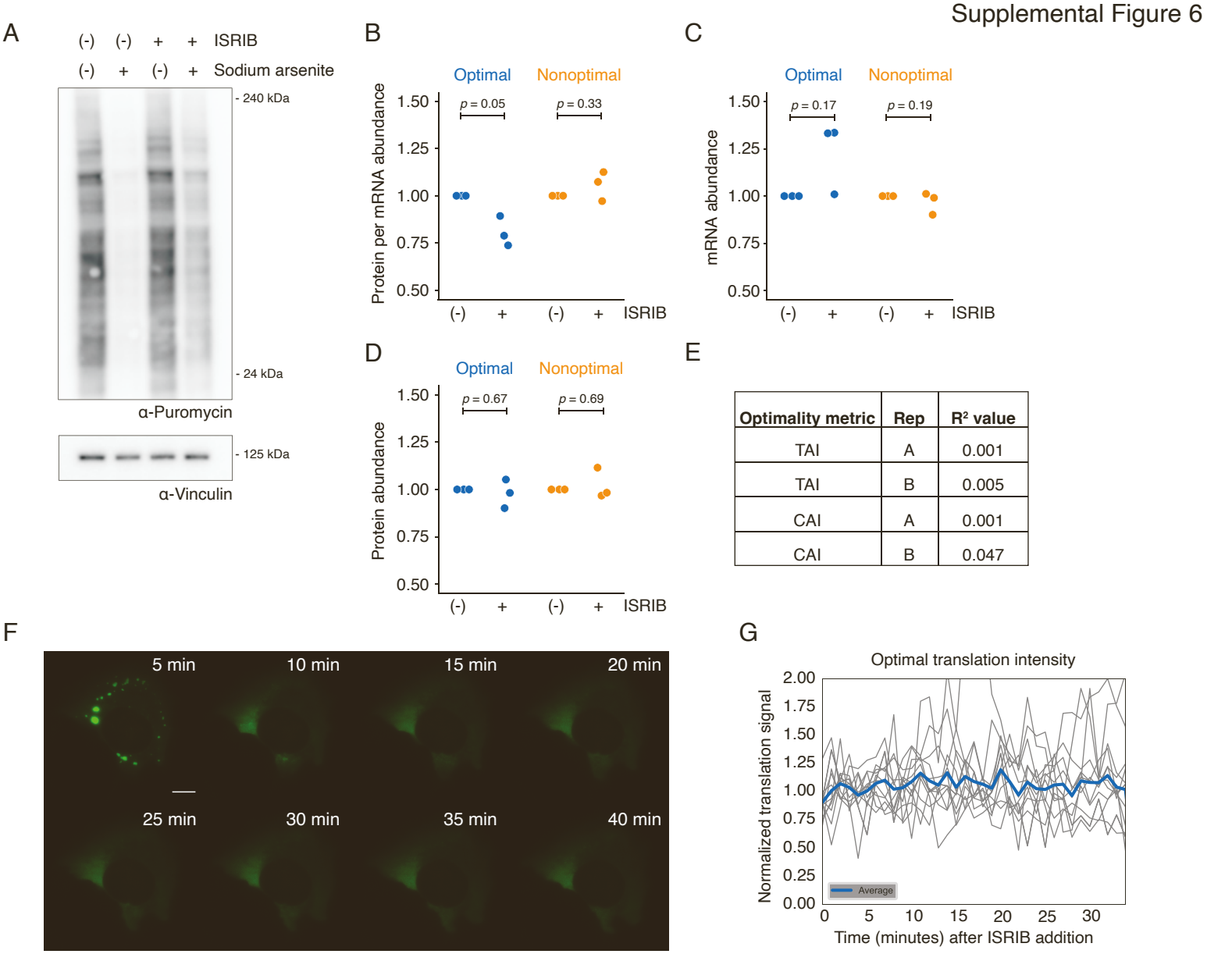


Figure S5. Reduced translational efficiency continues within the body of the nonoptimal reporter, and eIF4E/eIF4G1 can be successfully immunoprecipitated [Related to Figure 5].

(A) The translational efficiency of co-transfected *Renilla* luciferase with dual-tagged reporters was not different between the conditions. Translational efficiency was defined as Ribo-seq reads relative to RNA-seq abundance. *Renilla* luciferase co-transfected with the optimized dual-tagged firefly luciferase reporter was normalized to one. *P*-value was determined by paired Student's t-test; *n*=3. **(B)** Nonoptimal codons reduce ribosome association. Shown is the translational efficiency (defined as Ribo-seq reads relative to RNA-seq abundance) for the firefly luciferase coding region and C-terminal GFP, from the dual-tagged reporters. Values are normalized to the co-transfected *Renilla* luciferase control, and the optimal reporter was normalized to one. *P*-values were determined by paired Student's t-tests; *n*=3. **(C)** eIF4E was immunoprecipitated in both samples. Western blotting was performed, confirming successful eIF4E immunoprecipitation for both conditions: nonoptimal firefly luciferase (Non.), and optimal firefly luciferase (Opt.). Estimated size of eIF4E is 28 kDa. Differences in pulldown efficiency were normalized to *Renilla* co-transfection control. **(D)** As in C, except for eIF4G1 RNA immunoprecipitations. Estimated size of eIF4G1 is 220 kDa. Differences in pulldown efficiency were normalized to *Renilla* co-transfection control.



Optimality metric	Rep	R^2 value
TAI	A	0.001
TAI	B	0.005
CAI	A	0.001
CAI	B	0.047

Figure S6: Small molecule ISRIB does not rescue translational efficiency of nonoptimal transcripts [Related to Figure 5].

(A) ISRIB can rescue translation in sodium arsenite-treated cells after 24 hours of treatment. HEK293T cells were treated with 500 nM of ISRIB for 24 hours, then 50 mM sodium arsenite was added for the last hour, along with 2 mM puromycin for the last ten minutes. Shown is a western blot probing for total puromycin incorporation. **(B)** ISRIB treatment may reduce the translational efficiency of optimized firefly luciferase reporter. Data are replotted from the experiment shown in Figure 5B, except translational efficiency is now normalized to DMSO-treated cells; *n*=3. **(C)** ISRIB treatment may increase the mRNA abundance of the optimal firefly luciferase reporter. Data are replotted from the experiment shown in Figure 5B; transcript abundance is normalized to DMSO-treated cells; *n*=3. **(D)** ISRIB treatment does not impact the protein activity of firefly luciferase reporters. Data are replotted from the experiment shown in Figure 5B; protein activity is normalized to DMSO-treated cells; *n*=3. **(E)** ISRIB treatment does not change the translational efficiency in an optimality-dependent manner, regardless of the metric used. Shown is a table with R^2 values for how well changes in translational efficiency, transcriptome-wide, correlate with optimality scores [2]. Scale bar, 10 μM. **(F)** ISRIB treatment quickly disassembles stress granules caused by sodium arsenite. Shown are movie stills from a time course in U2OS cells; ISRIB was added immediately following time = 5 min. Stress granules are labeled in green (cells were transfected with GFP-G3BP1 expressing plasmid). **(G)** ISRIB treatment does not change ribosome occupancy on the optimal reporter mRNA. Each line denotes the average normalized translation signal (ribosome occupancy) per cell (*n*=13). **(H)** ISRIB treatment does not change ribosome occupancy on the nonoptimal reporter mRNA. Data are shown as in S6G; *n*=17.

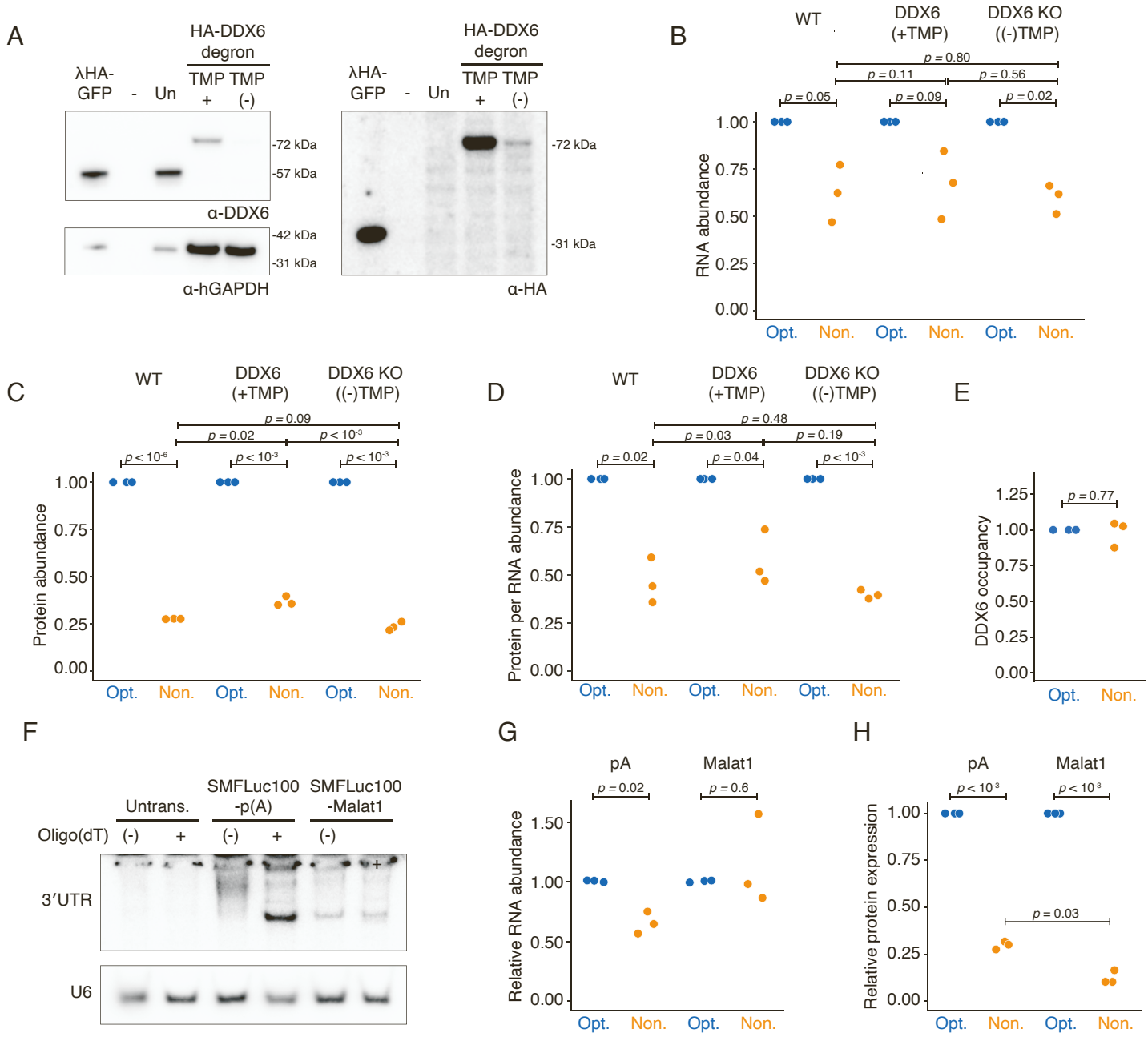


Figure S7: DDX6 does not impact gene expression of nonoptimal mRNAs, Malat1 reporters are indeed missing a poly(A) tail, and the Malat1 triple helix stabilizes nonoptimal reporter transcript levels while repressing nonoptimal protein expression [Related to Figure 6].

(A) Endogenously DHFR-tagged DDX6 protein in HEK293T cells is effectively depleted following TMP washout. From left to right: 1) cells transiently expressing λHA-GFP (~34 kDa), 2) skipped lane/ladder, 3) untransfected cells, 4) HA-DDX6-eDHFR cells (+)TMP (~72 kDa), 5) HA-DDX6 degron cells following TMP washout. hGAPDH = loading control. **(B)** DDX6 does not impact mRNA levels of nonoptimal SM-tagged firefly luciferase reporter. Shown are relative reporter mRNA expression from unmodified HEK293T cells (WT), HA-DDX6-eDHFR cells (+)TMP, and HA-DDX6-eDHFR cells (-)TMP. RNA values were determined by RT-qPCR (*p*-value = Student's t-test; *n*=3) **(C)** DDX6 does not impact the relative protein activity of nonoptimal SM-tagged firefly luciferase reporter (dual luciferase assay; *p*-value = Student's t-test; *n*=3). **(D)** DDX6 does not impact the translational efficiency of nonoptimal SM-tagged firefly luciferase reporter. Plotted are protein per mRNA levels from values shown in S7B and S7C. **(E)** Nonoptimal codons have no effect on DDX6 binding. DDX6 RIPs were performed (*p*-value = Student's t-test; *n*=3) **(F)** Reporters ending in Malat1 triple helix do not have a poly(A) tail. Shown is a northern blot with RNA from HEK293T cells: untransfected (lanes 1 & 2), expressing the SM-tagged firefly luciferase reporter ending in a poly(A) tail (lanes 3 & 4), and expressing the same reporters but ending in a Malat1 triple helix (lanes 5 & 6). To total RNA samples, "RNase H" DNA probe was annealed to the beginning of the reporter 3'UTR, along with oligo(dT) to appropriate samples, before digesting with RNase H. The "3'UTR_premalat1_as" probe annealed to the truncated 3'UTR product, while the U6 probe (loading control) annealed to the U6 small nuclear RNA. See Table S1 for probes. **(G)** Nonoptimal mRNA reporter levels are stabilized in the absence of deadenylation or a poly(A) tail. Relative mRNA abundance was determined by RT-qPCR (*p*-value = Student's t-test; *n*=3). **(H)** Despite rescued mRNA levels (S7G), nonoptimal protein levels are reduced when mRNA ends in Malat1 triple helical structure. Relative protein activity was determined with dual luciferase assays (*p*-values = Student's t-test; *n*=3).

[1] Juszkiewicz, S., and Hegde, R.S. (2017). Initiation of Quality Control during Poly(A) Translation Requires Site-Specific Ribosome Ubiquitination. Mol. Cell 65, 743-750.e4. 10.1016/j.molcel.2016.11.039.
[2] Sidrauski, C., McGeachy, A.M., Ingolia, N.T., and Walter, P. (2015). The small molecule ISRIB reverses the effects of eIF2α phosphorylation on translation and stress granule assembly. Elife 4. 10.7554/eLife.05033.

Table S1. Expanded list of RT-qPCR primers, CRISPR Cas9 guides, and probes used, as referred in STAR Methods Key Resource Table.

RT-qPCR primers	
Oligonucleotide name	**Sequence (5'->3')**
Fwd: spaghetti monster tag	5-GAAACCCGTACATGGGAACT-3
Rev: spaghetti monster tag	5-CACCACACCTCCATCTTCATAC-3
Fwd: optimal *Renilla* luc gene	5-GCAACTACAACGCCTACCT-3
Rev: optimal *Renilla* luc gene	5-CCACGAAGCTCTTGATGTACT-3
Fwd: optimal firefly luc gene	5-CCGACAGGGTTACGGTTTGA-3
Rev: optimal firefly luc gene	5-TAATCATCGGACCTCGCACG-3
Fwd: nonoptimal firefly luc gene	5- GGGGATGATAAGCCTGGAGC-3
Rev: nonoptimal firefly luc gene	5-CGGGTGCTACTTGATACCCC-3
Fwd: 3'UTR of pcDNA3.1 vector	5-GCCCGTTTAAACCCGCTGAT-3
Rev: 3'UTR of pcDNA3.1 vector	5-GGGAGGGGCAAACAACAGAT-3
Fwd: hGAPDH	5-CAAATTCCATGGCACCGTCA-3
Rev: hGAPDH	5-GACTCCACGACGTACTCAGC-3
Fwd: optimal *Drosophila* Renilla luc gene	5-AACGCCGCCTCTTCTTACTT-3
Rev: optimal *Drosophila* Renilla luc gene	5-CCAATCGTGACCGACGAAGA-3
Fwd: 3'UTR of *Drosophila* pAC5.1B vector	5-CCTAACCCTCTCCTCGGTCT-3
Rev: 3'UTR of *Drosophila* pAC5.1B vector	5-AGGCCTTAGAAGGCACAGTC-3

CRISPR Cas9 guides, etc.	
Oligonucleotide name	**Sequence (5'->3')**
hCNOT3 KO guide 1	TCTTGCGGATGGCGTCAACG
hCNOT3 KO guide 2	GACTCTGAGCGTATTCGGTG
NT guide	ATCGTTTCCGCTTAACGGCG
DDX6 degron upstream homology	G*G*AGCAGCTGGGAACAGAAATTAAACCTATTCCGAGCAACAT TGATAAGAGCCTGTATGTGGCAGAATACCACAGCGAGCCTGTAG AAGATGAGAAACCT ggaggcggttacccatac
DDX6 degron downstream homology	c*t*attatatatggtgtgacatcaaatttcccccaggaaagaagattttgatttccct catttaaaggttcctcttagctgttctgtcagggacgtacATGCTTG gtcgactgatcataatcagc
DDX6 degron guide	AGGGACGTACATGCTTGTTA
Fwd: DDX6 3' end screen	AGTATTGAGGAGCAGCTGGG
Rev: DDX6 3' end screen	TGAGACTACAACCTCCCACA

Northern probes	
Oligonucleotide name	**Sequence (5'->3')**
RNase H probe (annealed to upstream 3'UTR sequence, to cleave using RNase H)	5-CAGAATTCCACCACACTGGAC-3
3'UTR_premalat_as Northern probe	5-GTTTAAACGGGCCCTCTAGACTCGAGCGGC-3
U6 Northern probe	5-GCTAATCTTCTCTGTATCGTTCCAATTTTAGTATATGTGCTGCCG-3